

## Variant Surface Antigens of *Giardia lamblia* Are Associated with the Presence of a Thick Cell Coat: Thin Section and Label Fracture Immunocytochemistry Survey

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*Giardia lamblia* undergoes surface antigenic variation. The ultrastructural location of antigens on four different variants was studied by label fracture and immunocytochemistry with four monoclonal antibodies (MAbs), each of which recognized the predominant variant in a particular clone. Each *Giardia* clone and its reacting MAb showed similar findings. The entire surface of the organism was covered by a surface coat which contained the variant surface protein. The surface coat was densely and uniformly labeled. Unreacting MAbs failed to label the surface. Label-fractured *Giardia* trophozoites exposed to reactive MAb revealed a planar distribution of reactivity with no discernible relationship to visualized intramembranous particles. Unexpectedly, some *Giardia* trophozoites lacked a surface coat and consequently failed to react with the appropriate MAb. The biological relevance of coatless *Giardia* trophozoites is unknown. These findings localize the variant antigens to the surface coat of the parasite and identify a minority of the population which lacks a surface coat.

*Giardia lamblia* is a pear-shaped protozoan parasite which causes both endemic and epidemic diarrheal illness worldwide. Giardiasis is the most common human parasitic disease in the United States (24). After the host ingests a relatively resistant cyst form, the resultant motile trophozoite multiplies in the small intestine. Cysts form during passage through the intestine and are excreted in the feces, sometimes in massive amounts. Infections are common wherever fecal contamination occurs and are particularly common in backpackers (4), homosexuals (31), travelers (6, 35), and institutionalized persons and children attending day-care centers (5, 12). Large epidemics have occurred following the contamination of water reservoirs (8). In developing areas, infections of children approach 100% by 2 years of age (13).

Despite the increasingly recognized importance of the parasite, relatively little is known about the biology, biochemistry, and antigenic makeup of *G. lamblia* or about the host's immune response to infection. Recent studies have shown that *G. lamblia* undergoes surface antigenic variation (1, 14, 16). Clones derived from a single organism produce trophozoites possessing other surface antigens (16). The ability to express certain epitopes is characteristic of the parent isolate and therefore appears to be genetically determined (18). The surface location of the variant surface antigens is indicated by surface fluorescence after indirect and direct fluorescent antibody tests and by the fact that most antibodies directed to these antigens cause almost immediate cessation of flagellar and parasite motility, leading to death of the parasite (15). Isolates differ in both the rate of antigenic variation (17) and the epitopes they are able to express (18). In most instances, this is due to lack of the gene encoding the specific epitope (18). Although only a limited number of variants have been studied, they characteristically are cysteine rich (2) and show frequent Cys-X-

X-Cys motifs (1). Antigenic variation also occurs in vivo and has been found in experimentally infected humans (19, 20), mice (11), and gerbils (3). The biological relevance of this phenomenon is unclear, but recent studies indicate that certain variant surface antigens protect the parasite from host digestive proteases (21).

In this study, we used label fracture (30) and thin-section immunogold cytochemistry to show that four monoclonal antibodies (MAbs) directed against epitopes characteristic of different clonally derived trophozoite populations exclusively label the cell sets against which they are directed. In all cases, labeling of the variant antigen by its MAb was intense and uniformly distributed over the entire surface of the cell. We report also that while the vast majority of cells were strongly labeled, a significant minority was not labeled. Thin-section electron microscopy showed that in these unlabeled cells the cell coat was absent.

### MATERIALS AND METHODS

**Parasites and MAbs.** The origins of the isolates and clones used as well as the reactivities of MAbs have been described previously (2, 15). Briefly, four sets of clones whose surface antigens reacted with different MAbs were used. Clones WB-A6-6E7S, WB-A6-5C1S, GS/M-H7, and B6 react with MAbs 6E7, 5C1, G10/4, and 3F6, respectively. Each of the four MAbs reacted with the predominant variant surface antigen of four different clones. More than 90% of the clones reacted with one particular MAb and less than 1% reacted with any of the other MAbs. Ascitic fluid was produced as described before and used without further purification (15).

**Fixation procedure.** For the labeling of the cell surface with the MAbs, trophozoites were fixed for 30 min at 4°C in a solution containing 4% formaldehyde (freshly prepared from paraformaldehyde) plus 0.5% glutaraldehyde in Dulbecco's phosphate-buffered saline (D-PBS), pH 7.0. For routine preparations, the cells were fixed in 2.5% glutaral-

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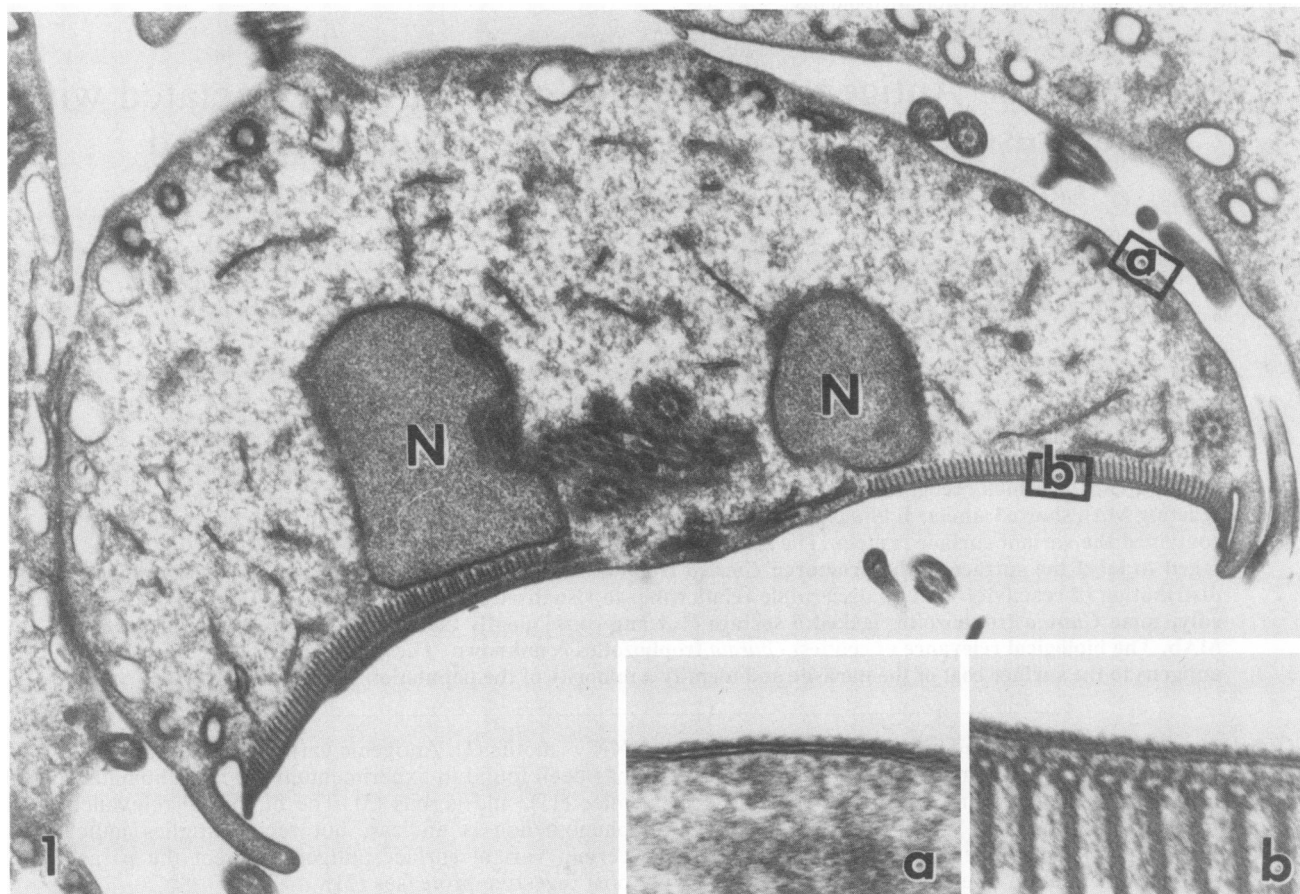


FIG. 1. General aspect of a *G. lamblia* trophozoite (WB-A6-6E7S). Note the thick cell coat entirely covering the dorsal (inset a) and ventral (inset b) parasite surface. N, nuclei. Magnification,  $\times 20,000$ ; insets,  $\times 140,000$ .

dehydrate in 0.1 M cacodylate buffer (pH 7.2) containing 5% sucrose and 5 mM  $\text{CaCl}_2$  for 1 h at room temperature (26).

**Immunocytochemistry.** After specific fixation for immunolabeling, the cells were washed several times in D-PBS at 4°C and each of the four clones was incubated with the four different ascitic fluids containing the respective MAb. The antibodies were diluted 1:200 with D-PBS–1% bovine serum albumin (D-PBS/BSA) (no. A-7030; Sigma Chemical Co., St. Louis, Mo.), and the organisms were incubated for 45 min at room temperature. After several washes in D-PBS/BSA, the trophozoites were incubated in 1:10 protein A conjugated with colloidal gold (10 to 15 nm) (EY Laboratories, Inc., San Mateo, Calif.). Control cells were incubated without MAbs or in the presence of nonimmune mouse serum.

**Thin sections.** After immunocytochemical labeling or routine fixation, trophozoites were postfixed in 0.1 M cacodylate buffer containing 5 mM  $\text{CaCl}_2$  and 0.8% potassium ferricyanide (26), dehydrated in acetone, and embedded in

Epon. Thin sections of the MAb-treated trophozoites were stained with lead citrate alone.

**Label fracture.** For label fracture (30), samples of trophozoites were collected after immunocytochemical labeling, washed in cacodylate buffer, and gradually impregnated in the same buffer containing 30% glycerol. Samples were then mounted in double-replica support disks, frozen in partially solidified nitrogen, and freeze fractured at  $-130^\circ\text{C}$ . After fracture, the specimens were immediately shadowed by platinum and carbon from electron guns and thawed in D-PBS. The replicas were washed overnight in distilled water, picked up on Formvar-coated grids, and observed with an electron microscope.

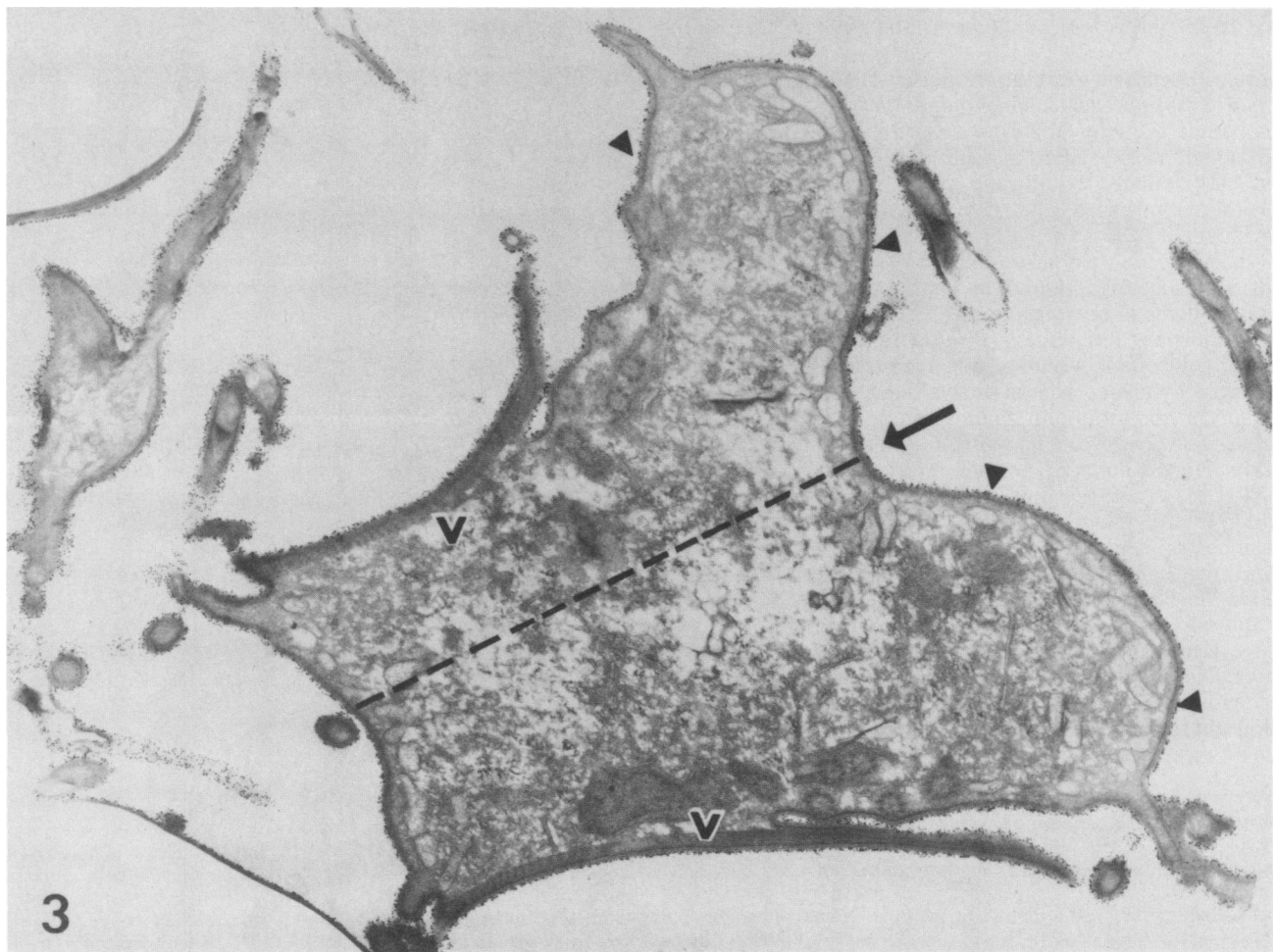
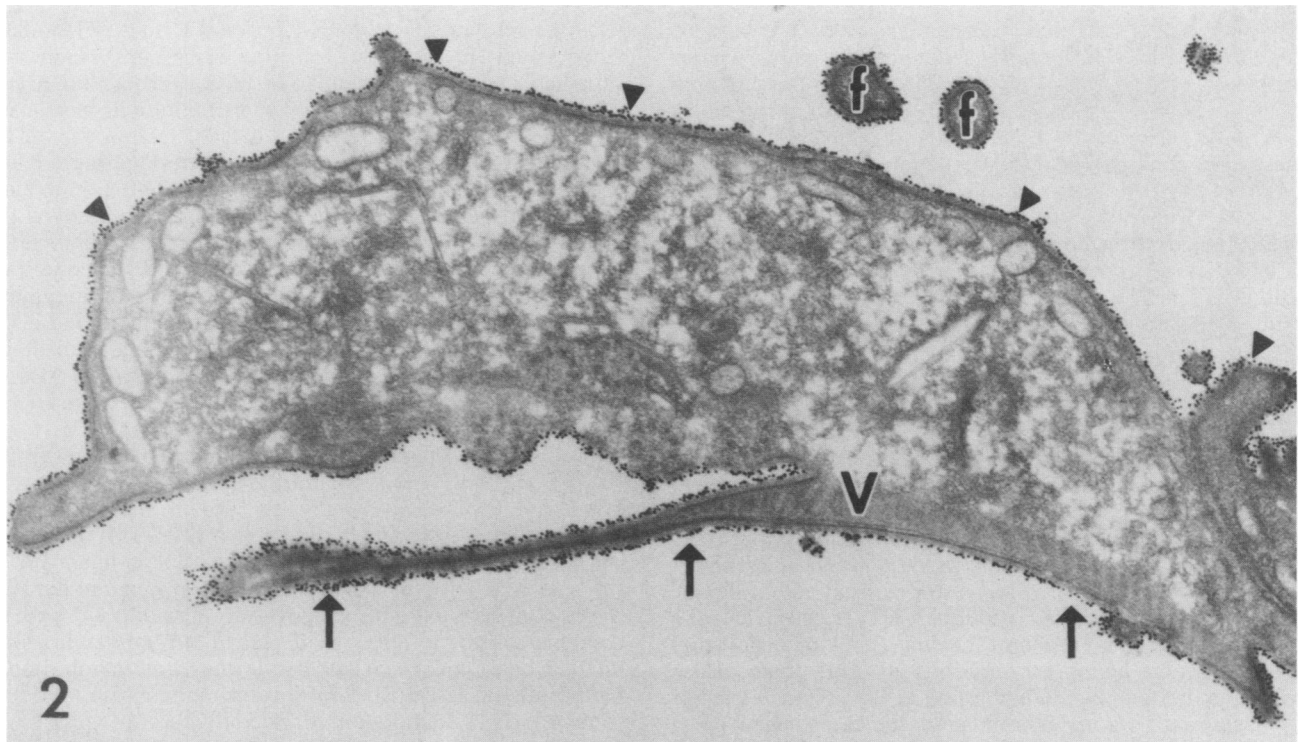
## RESULTS

Examination of thin sections confirmed previous ultrastructural descriptions (7, 9) of the trophozoites of *G.*

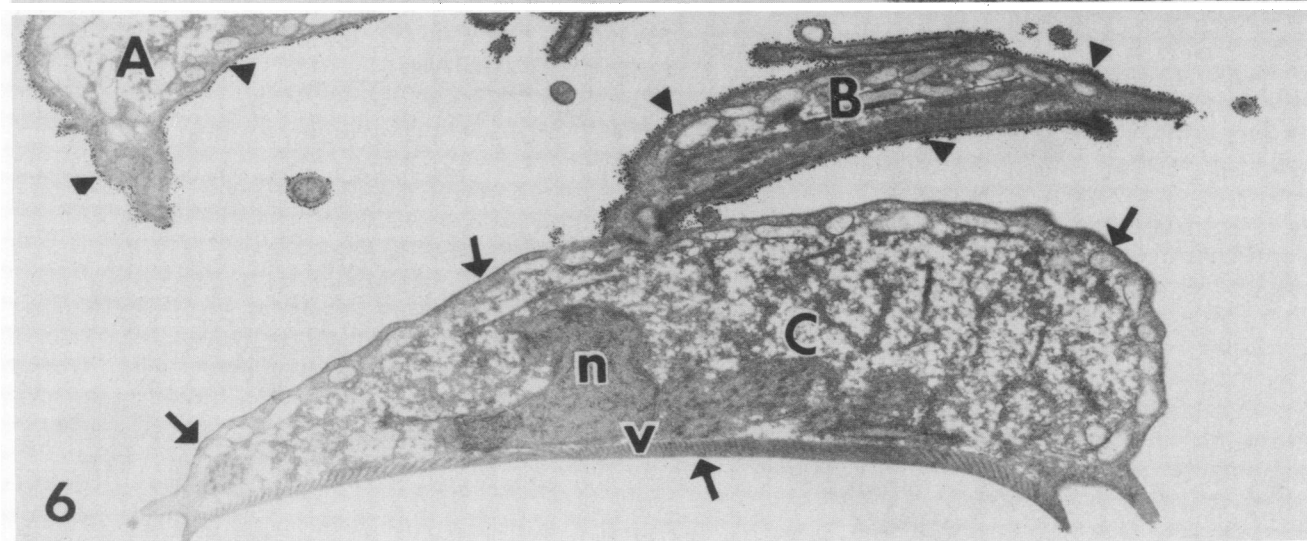
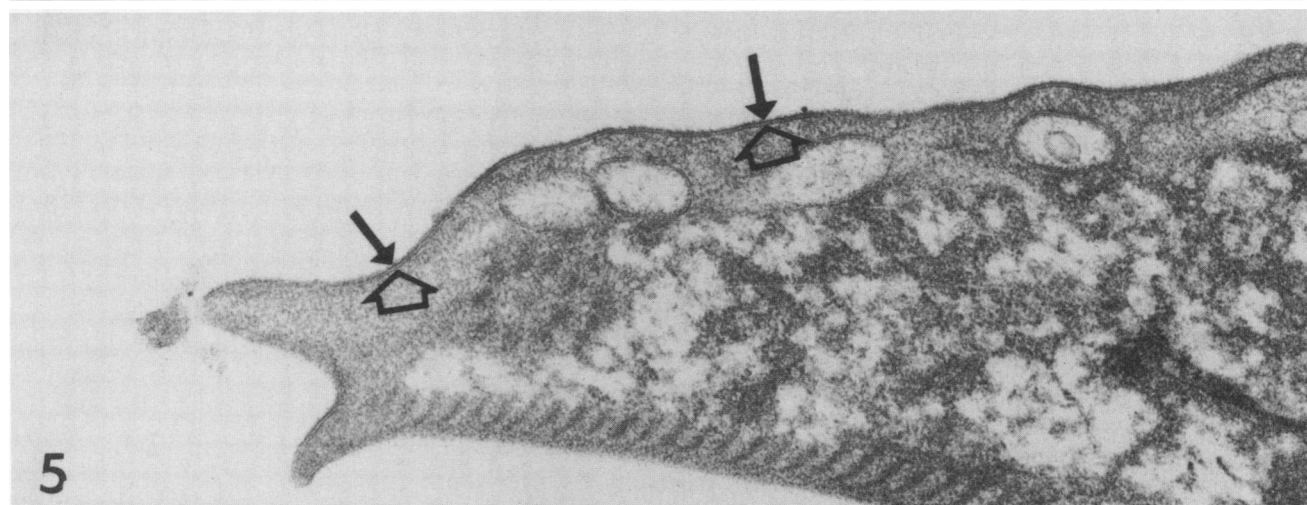
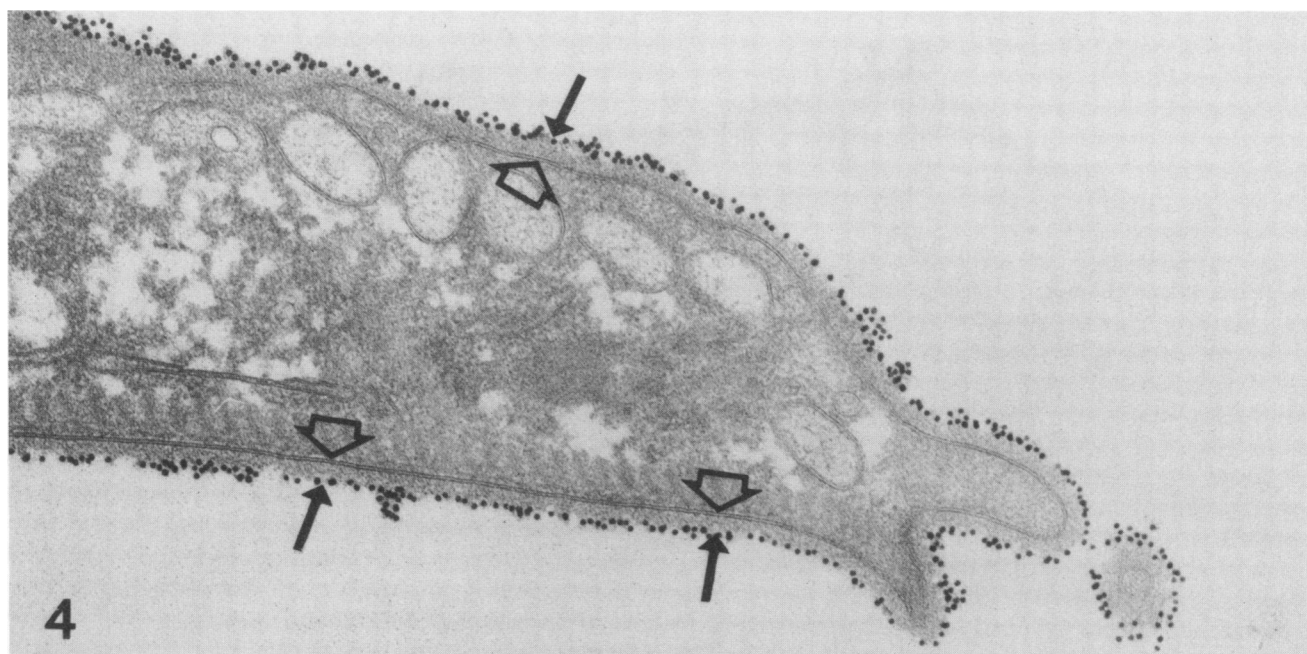
FIG. 2 and 3. Immunogold labeling of variant antigens. Gold particles (arrowheads) cover the entire cell surface of the WB-A6-5C1S and GS/M-H7 *Giardia* trophozoites treated with 5C1 and G10/4 MAbs, respectively, against the predominant variant surface antigen.

FIG. 2. There is no difference in the distribution of gold particles between the flagellar (f), dorsal (arrowheads), and ventral (arrows) cell surfaces. Magnification,  $\times 40,000$ .

FIG. 3. Dividing trophozoites with uniform distribution of gold particles (arrowheads) covering the entire cell coat including the ventral (V) surface. Dotted line, dividing trophozoites. Magnification,  $\times 26,000$ .







*lamblia* (Fig. 1). Of particular interest is a cell coat (thickness, approximately 18 nm) that surrounds the entire parasite, including the flagella. We found that this thick cell coat was absent in a minority of the parasites (about 10%).

Thin sections of labeled *G. lamblia* trophozoites showed that the variant surface antigens were localized on the cell coats of both undividing and dividing parasites (Fig. 2 and 3) and covered the entire surface of the parasite. An identical pattern was seen with each clone-MAb set. WB-A6-6E7S after exposure to MAb 6E7 showed dense homogeneous labeling of gold particles sitting on top of the cell coat (Fig. 4). No area appeared unlabeled. In contrast, the same organism exposed to another MAb (MAb G10/4) was labeled by very few gold particles (data not shown). Trophozoites which lacked the thick cell coat were not labeled (Fig. 5 and 6).

Freeze fracture splits the bilayered continuum of biological membrane, revealing intramembranous particles (IMPs), which represent integral membrane proteins. In all trophozoites, the IMPs were uniformly distributed along the plane of the membrane (data not shown). No differences were detected among the different *Giardia* clone-MAb sets.

Label-fractured *Giardia* trophozoites exposed to reactive MAbs illustrated the planar distribution of surface antigens in both dorsal and ventral cell surfaces (Fig. 7 and 8). The pattern of distribution was uniform; there was no discernible relationship between the distribution of the IMPs and that of the gold particles (Fig. 8). Immunogold labeling was also observed along the exterior rim of the cross-fractured membrane (Fig. 7, arrowheads). Here, the gold particles were separated from the edge of the fracture face by a distance of about 25 nm. This distance represents the aggregate thickness of the fractured cell membrane, the glycocalyx, and the intervening antibody-protein A.

Label fracture of trophozoites without the thick cell coat showed an absence of gold particles over the fractured membrane (Fig. 9).

Occasionally, an uneven pattern characterized by small aggregates of gold particles was observed (Fig. 10). There were no differences among the clone-MAb sets. Control clones exposed to nonreactive MAbs had few gold particles on their surfaces.

Some cells showed patches devoid of gold particles (Fig. 11 and 12). We thought these bare patches were artifactual and may have resulted from MAb-induced cell agglutination or from flagellae that were opposed against the cell surface during the labeling procedure.

## DISCUSSION

The main finding of this study is the localization of the variant surface antigens to the cell coat of the organism. Not only was the cell coat uniformly and densely labeled, the labeling was absent in a minority of cells in which the cell coat was absent. Our observations confirm previous immunofluorescence studies showing that the variant antigens

covered the entire cell surface (15). Their presence on the surface agrees with a number of other findings, including those of direct and indirect fluorescence studies of live organisms, studies of cytotoxicity and agglutination by MAbs, surface radiolabeling studies, and studies of shedding of variant antigens into the medium (reviewed in reference 14).

The lack of a visible cell coat that we consistently noted in a minority of the organisms was not expected. The absence of labeling showed that these cells do not present specific variant antigens. Why most trophozoites have a thick labeled cell coat and others do not is not clear. In another protozoan, *Leishmania major*, the acquisition of the high-molecular-weight lipophosphoglycan by the fully differentiated infective metacyclic promastigotes is reflected in a thicker cell coat (25, 29). Also, the three main developmental stages of *Trypanosoma cruzi* are distinguished by the morphology of the cell coat (27, 28). Possibly, unlabeled cells represent a particular stage of the life cycle, for instance, organisms in the process of changing surface antigens. Further investigation may clarify this aspect.

We found no differences among the MAb-variant antigen sets in either localization or pattern of distribution on the parasite surface. This supports other evidence indicating that these antigens indeed undergo sequential loss and replacement (16). In addition, we found no evidence to suggest the presence of more than one variant on the surface of an organism at one time. Few or no gold particles were seen in organisms labeled with nonspecific MAb-variant antigen, a labeling pattern similar to that observed in controls.

The pattern of the variant antigens over the cell surface was for the most part homogeneous and uniform, but in some cells, the gold particles were unevenly distributed and appeared to form small aggregates. In these cells, the pattern of distribution of IMPs was not altered. At first, this suggests that, at least in those cells, the surface sites labeled by the MAbs do not correspond to integral proteins. However, label fracture and fracture-flipped observations of capping phenomena in human lymphocytes indicate that integral membrane proteins may aggregate in small, defined domains without alteration in the overall distribution of IMPs (22, 23). This is because the inflow of specific proteins into one aggregate is made contemporaneously with outflow of the integral membrane proteins, therefore conserving the overall concentration of integral membrane proteins along the plane of the membrane. Our observations cannot establish whether or not the surface antigens that were labeled correspond to integral or peripheral membrane proteins. Previous studies (15) showed that these variant antigens are released by the trophozoites into the culture medium. Interestingly, MAbs to these variant antigens cause immobility and death independent of complement; this may be due to self-agglutination and inhibition of a vital function(s).

Wheat germ agglutinin (WGA)-reacting material was reported to exist on the surface of *G. lamblia* (33). However, the variant surface antigens used in the present study failed

FIG. 4-6. Immunolabeling of variant antigens (WB-A6-6E7S trophozoites treated with MAb 6E7 against the predominant variant surface antigen). Open arrows, plasma membrane. N, nucleus; V, ventral surface.

FIG. 4. High magnification of the cell surface of a trophozoite showing gold particles sitting atop the thick cell coat (solid arrows). Magnification,  $\times 80,000$ .

FIG. 5. Trophozoite exemplifying the minority population without gold labeling characteristic of the presence of the predominant variant antigen. Note the absence of the thick cell coat (solid arrows). Magnification,  $\times 80,000$ .

FIG. 6. General aspect of a population, with two trophozoites (A and B) immunolabeled by the predominant variant antigen (arrowheads) flanked by one (C) without gold labeling (arrows). Magnification,  $\times 16,000$ .

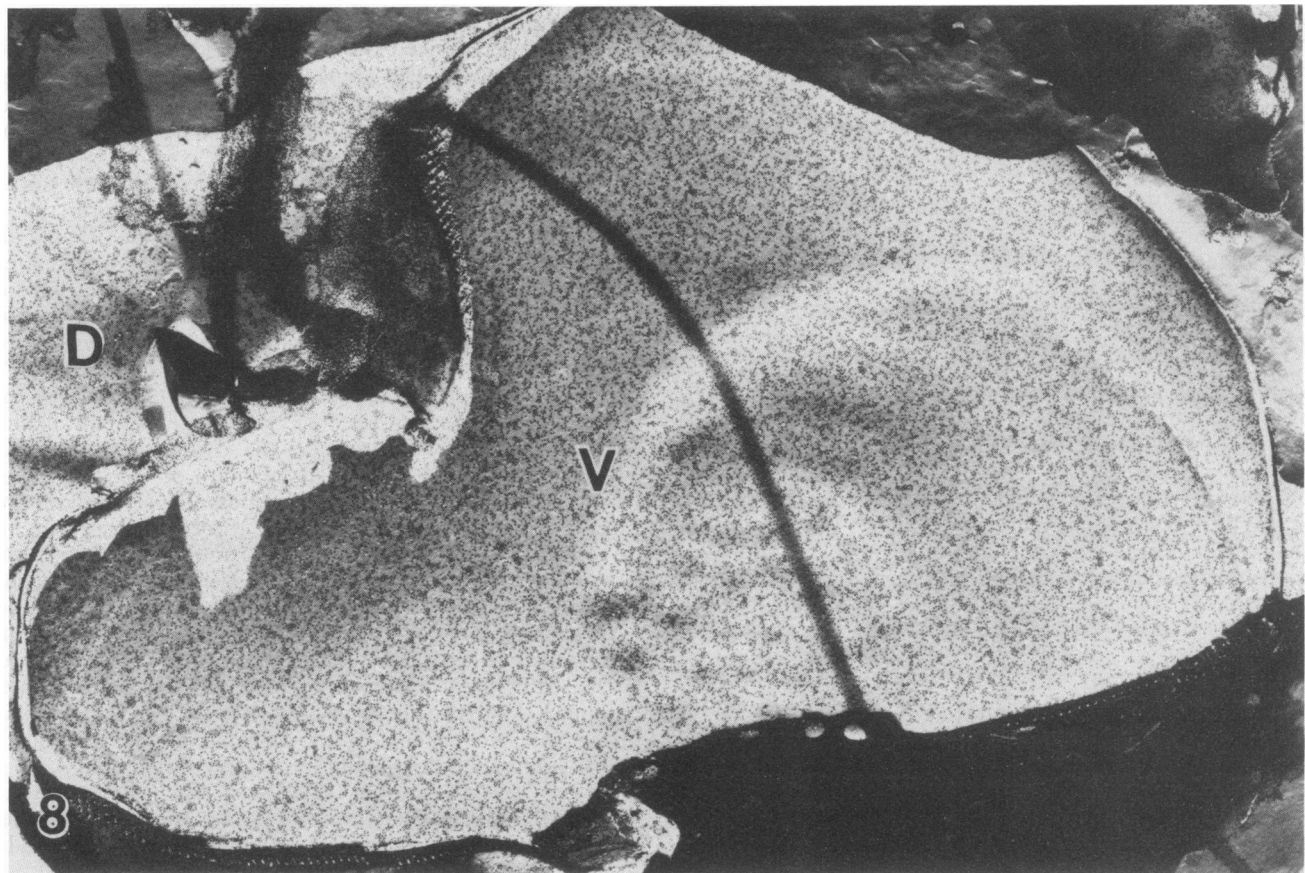
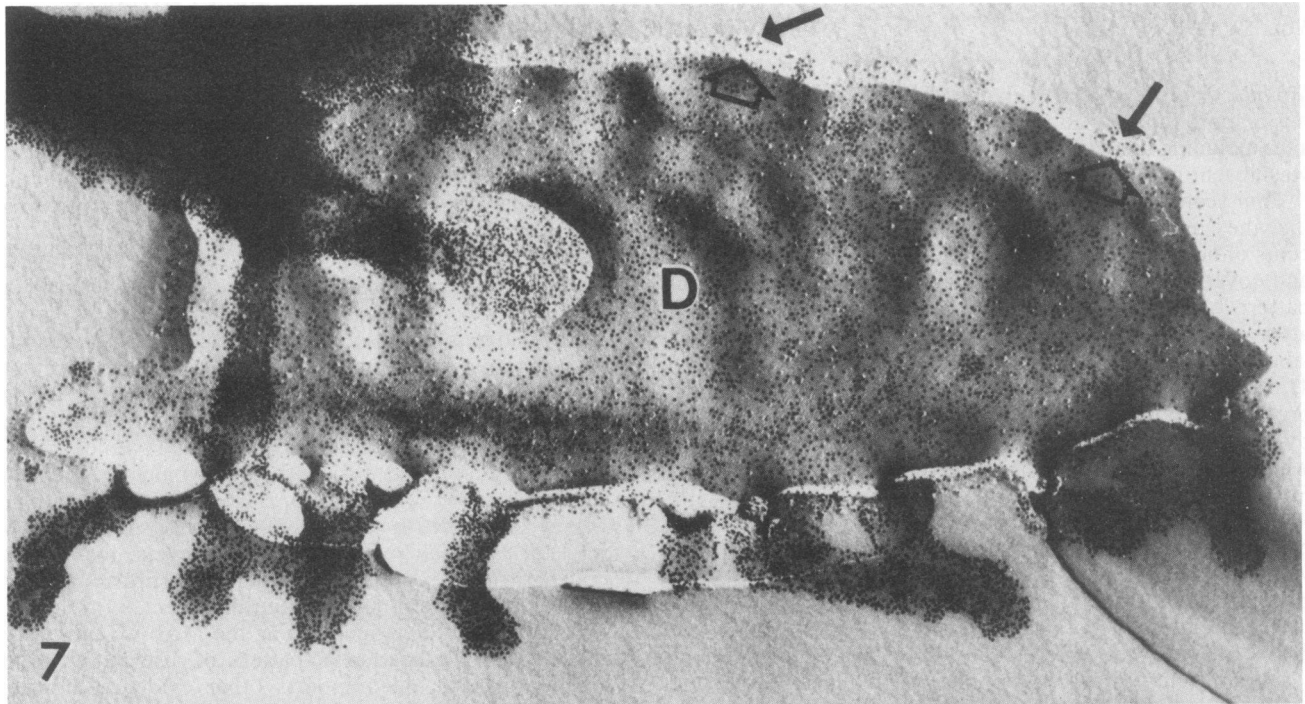


FIG. 7 and 8. Label fracture of immunolabeled *G. lamblia* (GS/M-H7 trophozoites treated with MAb G10/4 against the predominant variant surface antigen). Figures 7 and 8 show the dorsal (D) and ventral (V) surfaces of the trophozoites, respectively, with uniform distribution of gold particles. Note the gold particles on the exterior ring (closed and open arrows in Fig. 7) of the fractured membrane. Magnification: Fig. 7,  $\times 39,000$ ; Fig. 8,  $\times 28,000$ .

FIG. 9–12. Label fracture of immunolabeled *G. lamblia* (GS/M-H7 trophozoites treated with MAb G10/4 against the predominant variant surface antigen).

FIG. 9. Unlabeled trophozoite with no gold particles, including the exterior rim of the cross-fractured membrane (closed and open arrows). Note the traversing flagellum (F) of an immunolabeled trophozoite over the unlabeled trophozoite surface. Magnification,  $\times 40,000$ .



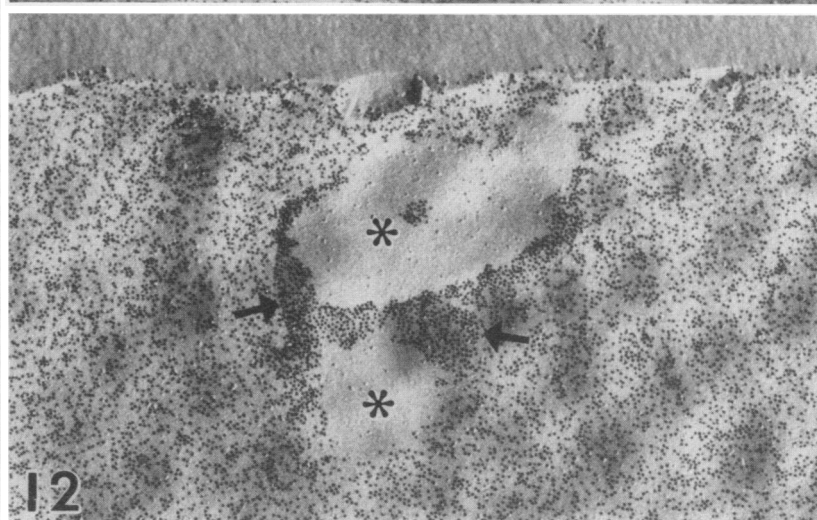
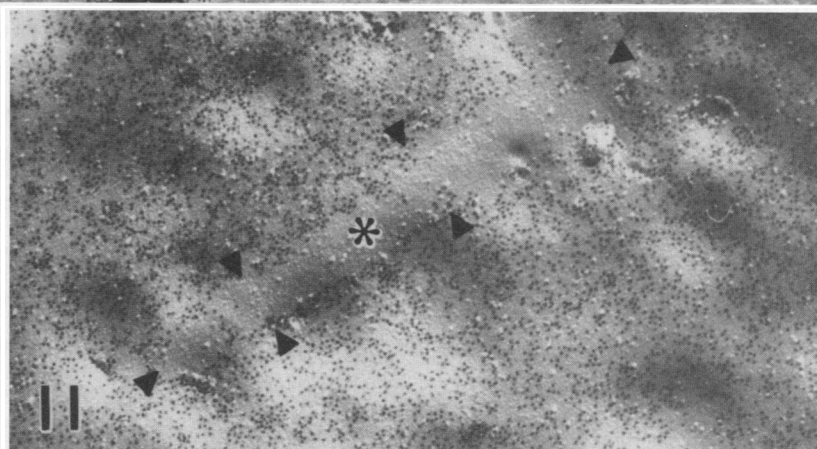
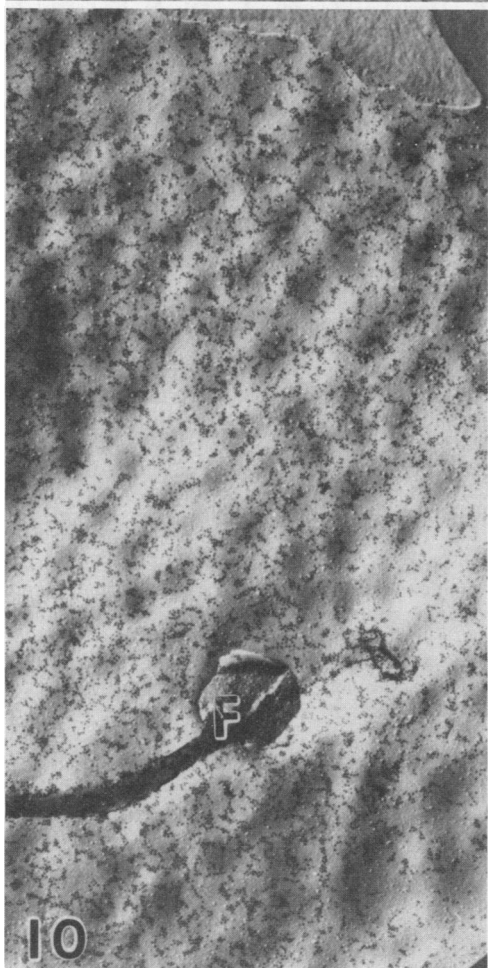
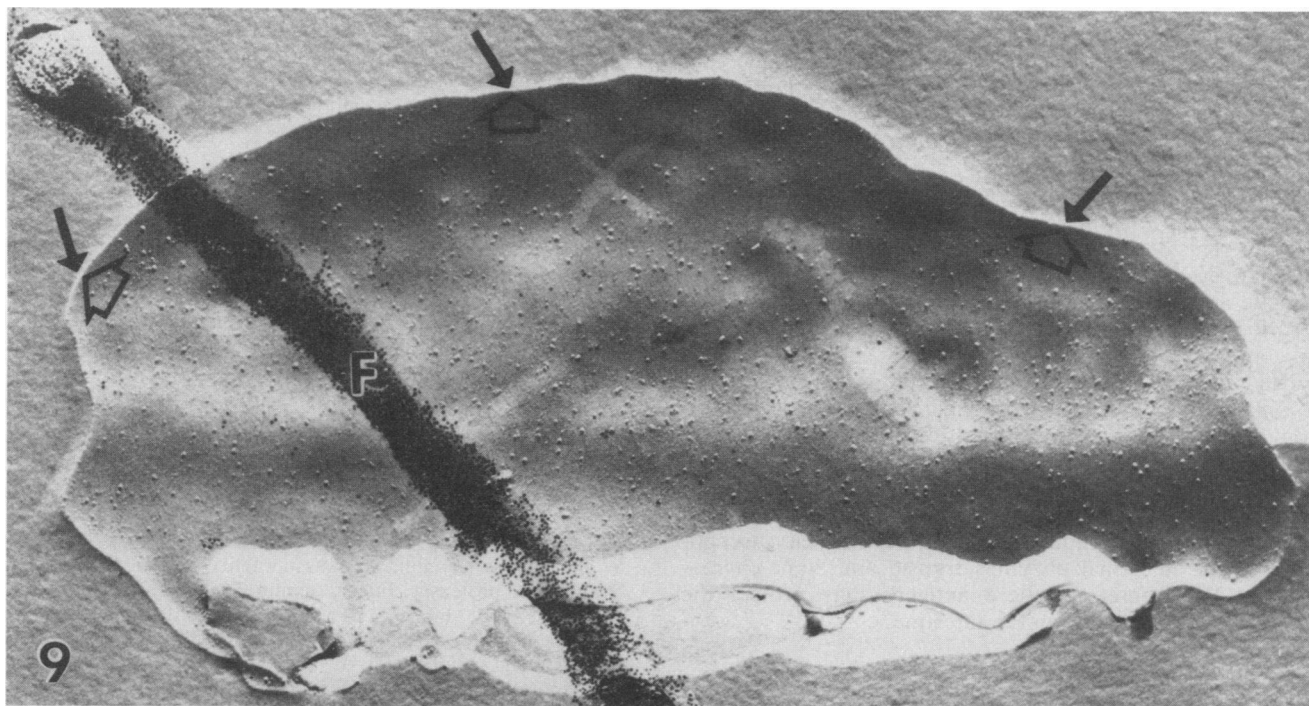


FIG. 10. Occasional trophozoite with uneven labeling pattern of small aggregates of gold particles. Magnification,  $\times 25,000$ .  
 FIG. 11 and 12. Infrequent linear (Fig. 11) or round (Fig. 12) patches (asterisks) devoid of gold particles on the cell surface. Magnification:  
 Fig. 11,  $\times 46,000$ ; Fig. 12,  $\times 40,000$ .

to react to WGA (personal observation). This suggests that WGA reactivity is due to other constituents of the cell coat or, alternatively, that WGA is reacting with *Giardia* trophozoites lacking a cell coat. Alternatively, some *Giardia* isolates or variants may have WGA-reacting sugars while others do not.

Gillin et al. (10) recently described a surface membrane antigen in *G. lamblia* which localized to the surface of parasite. This antigen was cysteine rich and contained multiple Cys-X-X-Cys motifs; antibody to it agglutinated the parasite and inhibited growth. The similarity to previously described surface variants suggests that it is a variant surface antigen.

The biological relevance of the variant surface antigens in *G. lamblia* is conjectural. Although immunological escape from the host's immune response is commonly thought to be of advantage to the organism, more than one cyclical change in surface antigens has not been documented in animal or human infections. Recent studies showed that *G. lamblia* isolates possessing unique variants differ in their susceptibility to the intestinal enzymes trypsin and alpha-chymotrypsin (21). Therefore, these antigens may protect the organism from digestion in the intestine.

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